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ORIGINAL ARTICLE

# Simultaneous quantification of tobacco alkaloids and major phase I metabolites by LC-MS/MS in human tissue

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**Abstract**
*Introduction* Insurance agencies might request laboratories to differentiate whether a deceased has been a smoker or not to decide about refunding of his nonsmoker rate. In this context, the question on a solid proof of tobacco alkaloids and major metabolites in tissues came up. Currently, an appropriate assay is still lacking to analyze tissue distribution in smokers or nonsmokers. Nicotine (NIC), nornicotine (NNIC), anatabine (ATB), anabasine (ABS), and myosmine (MYO) are naturally occurring alkaloids of the tobacco plant; most important phase I metabolites of NIC are cotinine (COT), norcotinine (NCOT), *trans*-3′-hydroxycotinine (HCOT), nicotine-*N*′-oxide (NNO), and cotinine-*N*-oxide (CNO). An analytical assay for their determination was developed and applied to five randomly selected autopsy cases.
*Methods* Homogenates using 500 mg aliquots of tissue samples were analyzed by liquid chromatography/tandem mass spectrometry following solid phase extraction. The method was validated according to current international guidelines.
*Results* NIC, COT, NCOT, ABS, ATB, and HCOT could be detected in all tissues under investigation. Highest NIC concentrations were observed in the lungs, whereas highest COT concentrations have been found in the liver. MYO was not detectable in any of the tissues under investigation.
*Conclusions* The assay is able to adequately separate isobaric analyte pairs such as NIC/ABS/NCOT and HCOT/CNO thus being suitable for the determination of tobacco alkaloids and their phase I metabolites from tissue. More autopsy cases as well as corresponding body fluids and hair samples will be investigated to differentiate smokers from nonsmokers.

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**Keywords** Tobacco alkaloids · Nicotine phase I metabolites · Liquid chromatography/tandem mass spectrometry · Tissue distribution

## Introduction

The affected person met a fatal accident. An opened cigarette box was found in his car damaged in the accident, which has been noticed by the life assurance company a few months later. Using exclusively this car and holding a policy of the more favorable nonsmoker rate, the life assurance company claimed additional payment from the next of kin. Therefore, determination of tobacco alkaloids from postmortem fluids was considered adequate to face this demand. However, only a few tissue samples, in particular from the liver, had still been available at the time of request. Moreover, data on tobacco alkaloids and major metabolites determined from human tissue to differentiate smokers from nonsmokers have not been published so far.

In tobacco products, nicotine (NIC) concentrations range from 0.6 to 1.8 % by weight [1]. Anabasine (ABS), anatabine (ATB), and nornicotine (NNIC) are the most abundant of the minor alkaloids accounting for less than 5 % of the nicotine concentration in tobacco products except cigars [2]. Further minor alkaloids are cotinine (COT), myosmine (MYO), and nicotine-*N*′-oxide (NNO) [3, 4].

Once being absorbed, NIC is extensively metabolized in man. Oxidation of NIC to COT is catalyzed by CYP2A6 and cytoplasmic aldehyde oxidase; about 70–80 % of NIC is converted to COT being the major metabolite. NNO is formed from about 4–7 % of the bioavailable NIC by a flavin-containing monooxygenase 3. *N*-Demethylation is a minor pathway of NIC biotransformation leading to NNIC. Formation of norcotinine (NCOT) might result from the oxidation of NNIC or demethylation of COT. COT is further metabolized by oxidation to cotinine-*N*-oxide (CNO) and by stereoselective hydroxylation to *trans*-3′-hydroxycotinine (HCOT) [4].

The present study was undertaken to develop and validate an assay by liquid chromatography-tandem mass spectrometry (LC-MS/MS) following solid-phase extraction (SPE) to determine tobacco alkaloids and their major phase I metabolites from human tissue.

## Experimental

### Materials

Standard solutions of (*S*)-NIC (1 mg/mL in methanol), (*R,S*)-NIC-d4 (100 μg/mL in acetonitrile), (*S*)-COT (1 mg/mL in methanol), (*R,S*)-COT-d3 (100 μg/mL in methanol), HCOT (1 mg/mL in methanol), (*R,S*)-NNIC (1 mg/mL in methanol), (*R,S*)-NCOT (1 mg/mL in methanol), and (*R*)-ABS-hydrochloride (1 mg/mL as free base in methanol) were purchased from Sigma-Aldrich (Steinheim, Germany). (*S*)-ATB, (*R,S*)-ATB-d4, (*R,S*)-ABS-d4, (S)-CNO, (*R,S*)-CNO-d3, MYO, MYO-d4, (2′*S*)-NNO, (*R,S*)-NNIC-d4, (*R,S*)-NCOT-d4, and HCOT-d3 were obtained as solids from Toronto Research Chemicals (North York, Ontario, Canada).

Ampuwa® water for injection was obtained from Fresenius Kabi (Bad Homburg, Germany). Ammonium acetate, dipotassium phosphate, sodium hydroxide, propan-2-ol, and methanol were supplied by Roth (Karlsruhe, Germany). Potassium chloride, monopotassium phosphate, formic acid, 25 % ammonium hydroxide, boric acid, 25 % hydrochloric acid, and dichloromethane were purchased from Merck (Darmstadt, Germany). Methanol was of HPLC grade; all other solvents and reagents were of reagent grade. Clean Screen SPE cartridges (130 mg, 3 mL) were from United Chemical Technologies (Bristol, Pennsylvania, USA). Bovine, porcine, and chicken livers obtained from a local supermarket were used for the preparation of calibration standards and quality controls (QCs). Authentic samples from the liver, lungs, kidneys, muscle, and brain were obtained from five randomly selected drug-related autopsy cases without information on smoking habits. The main purposes analyzing these samples were to establish an appropriate calibration range for each analyte due to hitherto missing data and to prove the method's applicability. Specimens had been stored at −20 °C until analyzed.

### Preparation and extraction of samples, calibrators, and quality control (QC) samples

Chopped tissue specimens were homogenized with an Ultra-Turrax after addition of water (1:4, *w/w*), stored at 4 °C overnight, and centrifuged (4300×*g*, 4 °C, 10 min). In addition, a homogenate of chicken liver was made up to prepare tissue control samples and calibration standards. For analysis of NIC and COT, six-point calibration curves ranged from 20 to 1000 ng/g; calibrator concentrations of MYO and HCOT were between 10 and 400 ng/g; NNIC, NCOT, and ABS calibrator concentrations ranged from 2.5 to 100 ng/g, for ATB, NNO, and CNO, calibrator concentrations were 10-fold lower. For details, see Table 1.

SPE columns were conditioned with 2 mL of methanol, water, and 1 mL of 75 mM phosphate buffer, pH 6.8, respectively. After addition of deuterated internal standards (IS), 2 mL of filtered calibration standards, QCs, and authentic samples were loaded onto the SPE cartridges by gravity flow. Columns were subsequently washed with 2×3 mL of water, 2 mL of 75 mM phosphate buffer, pH 6.8, and 2 mL of borate buffer, pH 8.5. Cartridges were vacuum dried for 30 min. One hundred μL of methanolic hydrochloric acid (methanol/HCl, 49:1, *v/v*) was added to silanized vials prior to elution of the analytes with freshly prepared 2 mL of dichloromethane/propan-2-ol/25 % ammonium hydroxide, 79:19:2 *v/v/v*. Eluates were taken to dryness under nitrogen at 40 °C and reconstituted in 50 μL of 10 mM ammonium acetate buffer, pH 5.0, containing 0.001 % formic acid.

### Liquid chromatography-tandem mass spectrometry (LC-MS/MS) conditions

LC was performed using an Agilent 1100 series HPLC system (Agilent, Waldbronn, Germany) consisting of an autosampler, a binary LC pump, and an additional isocratic pump for post-column injection. Chromatographic separation was achieved using a Luna C18 column (2.0×150 mm, 5-mm particle size, Phenomenex, Aschaffenburg, Germany) with a gradient system consisting of 10 mM ammonium acetate buffer, pH 5.0, with 0.001 % formic acid (mobile phase A) and methanol (mobile phase B) at a flow rate of 0.2 mL/min. The initial mobile phase condition was 0 % B which was increased linearly to 100 % B within 10 min. Then, 100 % B was held to elute hydrophobic compounds, decreased to initial mobile phase conditions after 15 min, and held for 15 min to re-equilibrate the LC column. Post-column injection of propan-2-ol at a flow rate of 0.05 mL/min increased peak intensities significantly for NNIC, NCOT, ABS, ATB, NNO, and CNO.

MS/MS analysis was performed using an API 4000 MS/MS (AB Sciex, Darmstadt, Germany) with a Turbo Ion ionization source operated in the positive ionization mode. Data were recorded in the multiple reaction monitoring mode, which are summarized in Table 1 for all analytes along with their respective retention time. Quantification was performed by comparing peak area ratios of target analytes to IS over all concentrations across the linear range.

### Method validation

Matrix effect, recovery, and process efficiency were investigated according to Matuszewski et al. [5] using spiked matrices from six different sources. Carry-over was checked as described by Bansal and DeStefano [6]. The lower limits of

**Table 1** Quality control (QC) concentrations [ng/g], retention times [min], and transitions [*m/z*] used for analysis

| Analyte | RT [min] | Mass transitions [*m/z*] | IS concentration [ng/g] and mass transition | Low QC [ng/g] | High QC [ng/g] |
|---|---|---|---|---|---|
| NIC | 14.4 | 163.3→130.1[a]<br>163.3→84.1 | NIC-d4; 200<br>167.2→134.1 | 50 | 500 |
| COT | 16.8 | 177.2→80.1[a]<br>177.2→97.9 | COT-d3; 200<br>180.2→80.1 | 50 | 500 |
| NNIC | 14.2 | 149.0→80.1[a]<br>149.0→132.1 | NNIC-d4; 20<br>153.3→84.0 | 5 | 50 |
| NCOT | 16.7 | 163.0→146.1[a]<br>163.0→135.1 | NCOT-d4; 20<br>167.0→139.0 | 5 | 50 |
| ABS | 16.4 | 163.0→117.0[a]<br>163.0→130.1 | ABS-d4; 20<br>167.2→121.1 | 5 | 50 |
| ATB | 16.3 | 161.0→144.0[a]<br>161.0→80.1 | ATB-d4; 2<br>165.2→148.0 | 0.5 | 5 |
| NNO | 8.1 | 179.0→84.2[a]<br>179.0→96.2 | NNIC-d4[b] | 0.5 | 5 |
| CNO | 15.0 | 193.0→96.1[a]<br>193.0→98.1 | CNO-d3; 2<br>196.0→96.1 | 0.5 | 5 |
| HCOT | 16.6 | 193.3→134.0[a]<br>193.0→80.1 | HCOT-d3; 80<br>196.0→80.1 | 20 | 200 |
| MYO | 17.3 | 147.2→105.0[a]<br>147.2→78.0 | MYO-d4; 80<br>151.1→109.0 | 20 | 200 |

*NIC* nicotine, *COT* cotinine, *NNIC* nornicotine, *NCOT* norcotinine, *ABS* anabasine, *ATB* anatabine, *NNO* nicotine-*N′*-oxide, *CNO* cotinine-*N*-oxide, *HCOT trans*-3′-OH-cotinine, *MYO* myosmine

[a] Transitions were used for quantification

[b] Deuterated NNO was not available

detection (LLOD) and quantification (LLOQ) were estimated from the calibration curves according to the International Conference on Harmonisation guideline for validation of analytical procedures [7]. Linearity of response was checked compliant with the Food and Drug Administration guideline for industry [8]. For imprecision experiments, QCs of low and high concentrations were pooled, aliquoted, and stored at −20 °C. Samples were both extracted and measured in duplicate on eight different days being compared to freshly made calibrators.

To assess on-instrument stability, six QCs at low and high concentration levels, respectively, were extracted, analyzed, and reanalyzed after 12 and 24 h. Freeze-thaw stability using six QCs at low and high concentration levels, respectively, was assessed for three freeze (23 h)-thaw (1 h) cycles. Analytes were considered stable if mean values of reanalyzed samples were within 85–115 % of mean values determined from the original samples [9].

## Results

### Method development

Although SPE proved to be superior to liquid/liquid extraction with regard to matrix effects, four aqueous washing steps were necessary to inhibit severe ion suppression. Diluted organic solvents, however, were unsuitable already eluting analytes from the cartridges thus decreasing extraction efficiency. Overall, extraction efficiencies of 50 % or better were achieved for all analytes except CNO at low and high QCs and HCOT at high QC.

Extracted ion chromatograms of a QC sample are shown in Fig. 1. A 30-min run was sufficient to elute all analytes and endogenous compounds yet necessary to adequately separate analytes such as NIC, NCOT, and ABS or HCOT and CNO sharing the same precursor ion and some identical fragment ions.

### Method validation

First experiments using bovine, porcine, and chicken liver specimens revealed that NIC, COT, NNIC, ABS, ATB, NNO, and HCOT were present in all blank samples. NIC concentrations were highest ranging from 8.3–27.3 ng/g. All other detectable analytes were present in amounts <5 ng/g. COT could be detected in all three tested blank matrices at concentrations <LLOQ. CNO was only present in trace amounts in bovine liver. Overall, concentrations were lowest in porcine and chicken livers but NIC concentrations were higher in porcine than in chicken liver (27 versus 15 ng/g).

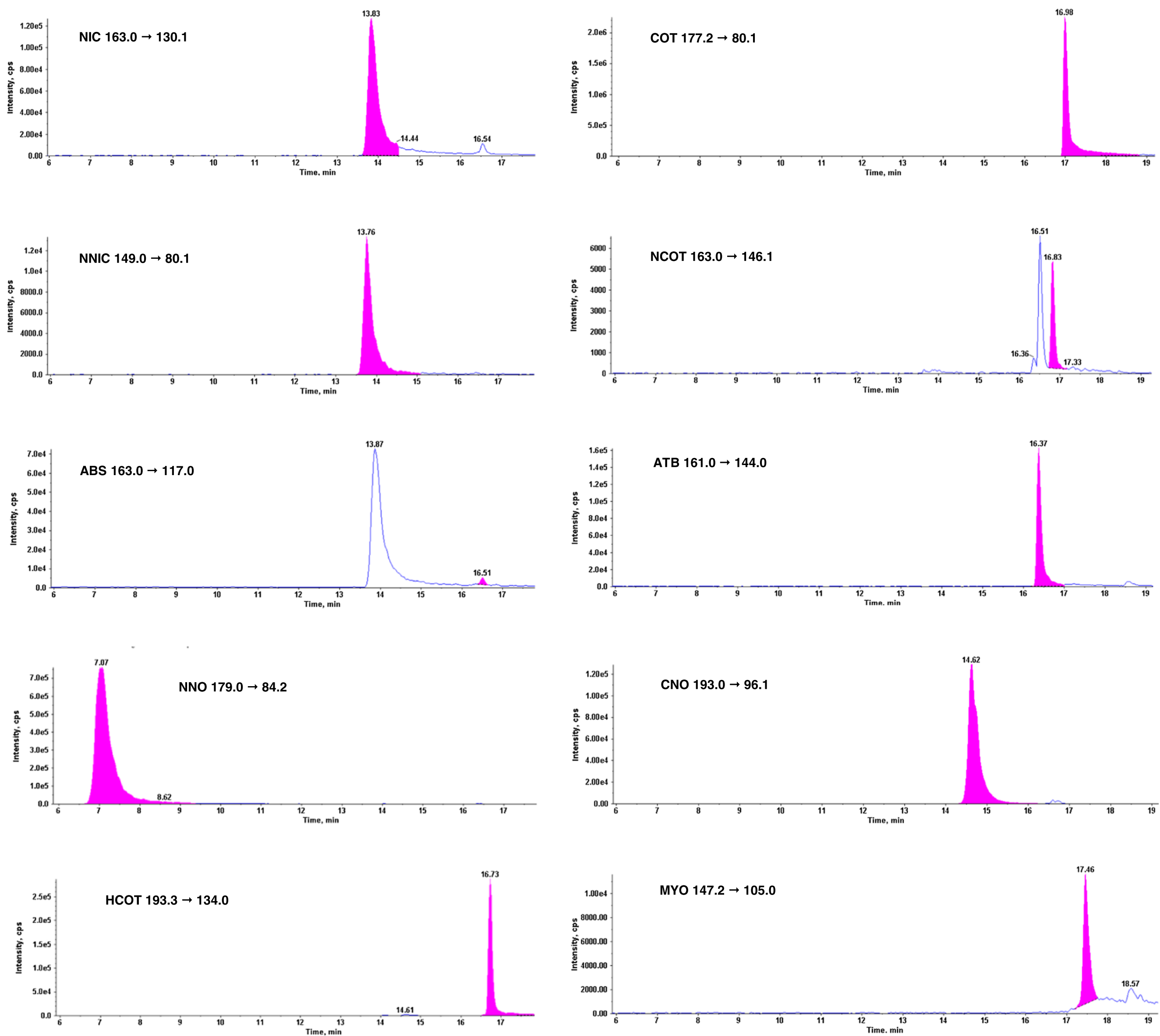


**Fig. 1** Target ion extracts of a chromatogram of a quality control sample (low) prepared in chicken liver homogenate. The *highlighted peaks* are the respective target analytes. Concentrations: nicotine (NIC) and cotinine (COT), 50 ng/g; nornicotine (NNIC) and anabasin (ABS), 5 ng/g; anatabine (ATB), nicotine-*N′*-oxide (NNO), and cotinine-N-oxide (CNO), 0.5 ng/g; trans-3′-OH-cotinine (HCOT) and myosmine (MYO), 20 ng/g

Chicken liver being always in stock was used for further experiments.

For validation and application purposes, five blank chicken liver samples fortified with respective IS were extracted in addition to calibrators. The peak area ratio of each calibration standard was adjusted by the mean peak area of NIC, COT, NNIC, ABS, ATB, NNO, or HCOT to its internal standard determined from chicken liver blanks; QC peak area ratios were corrected in the same manner.

Linearity of calibration lines was assessed using least square regression of quantities versus peak area ratios. Due to the broad calibration ranges, heteroscedasticity was observed. According to Almeida et al. [10], a weighted least square linear regression with a weighting factor of $1/x$ was applied, resulting in regression coefficients of ≥0.993. All calibration standards were within 10 % of the respective target concentration.

No significant ion suppression/enhancement (range 79–111 % low QC, 80–107 % high QC) or carry-over could be observed. For all analytes, 12-h-on-instrument stability could be proven at the low and high QCs; this was also true of the 24-h-on-instrument stability except for NNO at both the low and high QCs. The mean NNO concentration recovered after 24 h was 73 and 72 % at the low and high QCs, respectively.

NIC, COT, and ATB were stable at both QC levels following three freeze-thaw cycles; in addition, degradation of MYO

and CNO did not occur at the high QC. NNIC, NCOT, ABS, and NNO showed substantial degradation during three freeze-thaw cycles. At the low QC concentration level, 11–79 % of the initial mean concentration of NNIC, NCOT, ABS, NNO, CNO, and MYO could be recovered with NNIC being the most unstable analyte. At the high QC level, only 22–74 % of the initial concentration of NNIC, NCOT, ABS, and CNO were still present. Again, NNIC was most susceptible to freeze-thaw cycles. Interestingly, the mean ATB concentration increased by 22 and 39 % with regard to the mean initial peak at the low and high QC concentrations, respectively. Due to the unexpected effects of freezing and thawing on the analytes' stability, precision measurements had to be cut down on the determination of intraday precision. Further validation results are given in Table 2 and Fig. 2.

### Findings from human tissue

NIC, COT, NCOT, ABS, ATB, and HCOT could be detected in all tissues under investigation, whereas the N-oxides of NIC and COT were detectable in a few tissue specimens only, without exhibiting a distinct distribution pattern. Highest NIC concentrations were observed in the lungs, whereas highest COT concentrations were present in liver tissue. NIC concentrations were lowest in muscle tissue. In all tissue samples, ABS was about 10 to 30 times higher in concentration than ATB. The amount of HCOT determined from all tissues was highest among all metabolic products being formed from COT. MYO was not detectable in any of the authentic tissue samples. Concentrations are summarized in Table 3.

## Discussion

### Method development

A LC-MS/MS method for simultaneous analysis of tobacco alkaloids and major phase I metabolites in tissues has been established and validated following a rather sophisticated SPE procedure. Of the main analytical methodologies such as LC, GC, and immunoassays, LC-MS/MS appears most promising, allowing simultaneous determination of a range of analytes with varying physicochemical properties [11]. At present, LC-MS/MS assays have been developed to determine tobacco alkaloids from urine, plasma, oral fluid, and meconium [11–15].

NIC and/or COT have most frequently been measured and used as markers of nicotine and tobacco smoke exposure [11, 16]. There are only few methods including ABS and ATB [17, 18]. The assays of Miller et al. [13, 19], Piller et al. [11], and Vieira-Brock et al. [20] cover a spectrum of analytes similar to that of the present investigation, whereas other publications have considered NIC and COT, the respective desmethyl metabolites, and/or HCOT [12, 21].

The first problem that arises in the analysis of tobacco alkaloids and their metabolites in biological specimens is the availability of a matrix that contains no or minimum, reproducible amounts of the compounds being analyzed. This problem has hitherto not adequately been addressed. Exposure to tobacco alkaloids is not limited to the use of tobacco products and secondhand smoke. Tobacco alkaloids are also ingested with food, e.g., potatoes, tomatoes [22], and nuts [23] as well as rice, corn, and dairy products [24]. The presence of NIC and COT in forage and silage [25] might also be a

**Table 2** Validation data for the determination of tobacco alkaloids and their phase I metabolites in liver homogenate including the calibration range and coefficient of correlation (*r*), intraday precision, lower limits of detection (LLOD) and quantification (LLOQ) (ng/g), matrix effect (%), and extraction and process efficiencies (%)

| Analyte | Linear range (ng/g) and *r* | LLOD (ng/g) | LLOQ (ng/g) | Intraday precision (*n*=6, %) low QC, high QC | Extraction efficiency (*n*=6, %) low QC, high QC | Matrix effect (*n*=6, %) low QC, high QC | Process efficiency (*n*=6, %) low QC, high QC |
|---|---|---|---|---|---|---|---|
| NIC | 25–1000, *r*=1.0000 | 1.6 | 5.9 | 1.9, 1.6 | 93, 85 | 98, 96 | 91, 82 |
| COT | 25–1000 *r*=0.9999 | 2.2 | 7.9 | 8.6, 2.4 | 97, 86 | 101, 104 | 98, 89 |
| NNIC | 2.5–100 *r*=0.9991 | 0.1 | 0.4 | 3.3, 1.8 | 71, 51 | 90, 92 | 64, 47 |
| NCOT | 2.5–100 *r*=0.9938 | 0.5 | 1.9 | 4.3, 1.8 | 101, 80 | 90, 107 | 91, 86 |
| ABS | 2.5–100 *r*=0.9999 | 0.2 | 0.6 | 2.6, 3.0 | 130, 84 | 90, 100 | 117, 84 |
| ATB | 0.25–5 *r*=0.9999 | 0.05 | 0.19 | 2.6, 3.9 | 97, 97 | 111, 96 | 94, 130 |
| NNO | 0.25–10 *r*=0.9997 | 0.03 | 0.10 | 7.6, 6.1 | 116, 69 | 79, 80 | 93, 55 |
| CNO | 0.25–10 *r*=0.9992 | 0.07 | 0.26 | 2.5, 2.8 | 21, 10 | 96, 97 | 20, 9 |
| HCOT | 10–400 *r*=1.0000 | 1.3 | 4.9 | 6.7, 3.7 | 52, 33 | 86, 101 | 44, 33 |
| MYO | 10–400 *r*=1.0000 | 1.2 | 4.3 | 3.8, 2.4 | 100, 80 | 95, 99 | 95, 79 |

*NIC* nicotine, *COT* cotinine, *NNIC* nornicotine, *NCOT* norcotinine, *ABS* anabasine, *ATB* anatabine, *NNO* nicotine-*N′*-oxide, *CNO* cotinine-N-oxide, *HCOT trans*-3′-OH-cotinine, *MYO* myosmine

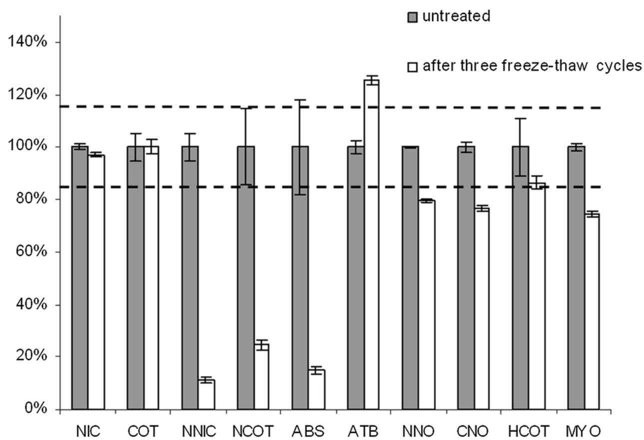


**Fig. 2** Stability of tobacco alkaloids and their phase I metabolites in liver homogenate ($n$=6) after three freeze-thaw cycles. The *dashed lines* indicate the ±15 % acceptance criterion, the *error bars* point out the standard deviation. *NIC* nicotine, *COT* cotinine, *NNIC* nornicotine, *NCOT* norcotinine, *ABS* anabasin, *ATB* anatabin, *NNO* nicotine-*N′*-oxide, *CNO* cotinine-*N*-oxide, *HCOT trans*-3′-OH-cotinine, *MYO* myosmine. Concentrations: NIC, COT 50 ng/g; NNIC, NCOT, ABS 5 ng/g; ATB, NNO, CNO 0.5 ng/g; HCOT, MYO 20 ng/g

plausible explanation for our findings in bovine, porcine, and chicken livers.

SPE proved to be superior over liquid/liquid extraction. Only Marclay and Saugy [26] published a method for the determination of NIC, COT, NNO, CNO, and HCOT from urine following liquid/liquid extraction. Due to unacceptably high matrix effects, liquid-liquid extraction cannot be deemed appropriate for the processing of tissue homogenates. Still, four washing steps had to be performed to reduce ion suppression or enhancement to values between 75 and 125 % for all analytes.

As already indicated above, ABS, NCOT, and CNO are not covered by most previously published studies. A LC method being unable to adequately separate isobaric analyte pairs such as NIC/ABS/NCOT and HCOT/CNO will give rise to an overestimation of NIC and HCOT, which are most often analyzed to prove smoking.

## Method validation

Reports on the determination of tobacco alkaloids and major phase I metabolites from tissue samples are scarce. Shakleya and Huestis [27] found slightly lower extraction efficiencies for NIC and COT after SPE from human brain homogenate, whereas a significantly higher extraction yield for HCOT of 82–99 % was reported compared to our study. The four washing steps might be a likely explanation, which, however, were necessary to end up with minimum and reproducible matrix-induced alterations of MS/MS signals in our assay. Shakleya and Huestis [27] observed erratic matrix suppression of 13–59 % for NIC, 18–75 % for COT, and 21–35 % for HCOT whereas matrix effects in the present study ranged from 96–98 % for NIC, 101–104 % for COT, and 86–101 % for HCOT.

Vieira-Brock et al. [20] reported on matrix effects in rat brain comparable to those of the present findings for all analytes except MYO, which was not included in their assay. Extraction efficiencies were also within the same range except HCOT and CNO, which were 82–89 % and 64–81 %, respectively, compared to 33–52 % for HCOT and 9–20 % for CNO in the present method. This may be due to two extraction steps using 5 % ammonium hydroxide in methanol, followed by a mixture of dichloromethane, propan-2-ol, and 25 % ammonium hydroxide [20]. Still, as in the present method, CNO had the lowest extraction efficiency.

Most frequently and most comprehensively also, stability of the analytes during the validation procedure has been tested in urine [11, 19, 28]. Piller et al. [11] found all analytes to be stable for 24 h at 10 °C when stored in an autosampler and through six freeze-thaw cycles without providing a criterion how stability has been assessed. Differences seen during on-instrument stability are likely due to different storage temperatures. Miller et al. [19] recovered 80–120 % of the initial concentration of all analytes in QC plasma and urine samples after three freeze-thaw cycles.

At present, data on the stability of the compounds under investigation in tissue homogenates are unavailable. Only Shakleya and Huestis [27] investigated the freeze-thaw stability of NIC, COT, and HCOT in human brain, observing a loss of less than 10 % of the initial concentration. In our study, these analytes were also stable through three freeze-thaw cycles. The different matrices may serve as a likely explanation of the degradation of NNIC, NCOT, and ABS in liver homogenate during freeze-thaw cycles which is in contrast to findings from urine and plasma.

Heavner et al. [28] observed no changes of the initial concentration for NIC, COT, and HCOT isolated from authentic urine samples of smokers through a 48-h-on-instrument stability test at ambient conditions. A further study reported on NIC, COT, NNIC, NCOT, ABS, ATB, NNO, CNO, and HCOT showing a maximum deviation of 20 % of the initial concentration after 72-h-on-instrument storage of extracts from plasma and urine QC samples at 4 °C. The amount of NNIC, NCOT, NNO, and HCOT in plasma decreased by 16–20 % at both concentration levels under investigation and by 7–16 % in urine QC samples stored at the same conditions [19]. The lower on-instrument stability of NNO in liver extracts compared to plasma or urine might be explained by the lower temperature of 4 °C in the experiments conducted by Miller et al. [19] compared to the present condition. Still, comparison of both studies is difficult due to different matrices. To limit substance loss during analysis exceeding 12 h, evaporated extracts should gradually be redissolved. Alternatively, the autosampler should operate at 4 °C.

**Table 3** Concentrations of tobacco alkaloids and phase I metabolites in randomly selected human tissue samples in ng/g

| Case no. material | NIC | COT | NNIC | NCOT | ABS | ATB | NNO | CNO | HCOT | MYO |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | | | |
| Lungs | 70 | 137 | 5.7 | 4.0 | 17 | 1.2 | nd | nd | 40 | nd |
| Kidneys | 1075 | 221 | 42.5 | 20.4 | 56 | 5.2 | 6.5 | 13.6 | 55 | nd |
| Liver | 107 | 456 | 2.0 | 3.1 | 19 | 1.4 | nd | nd | 41 | nd |
| Brain | 71 | 82 | nd | 3.8 | 17 | 2.4 | nd | nd | 32 | nd |
| Muscle | 52 | 84 | nd | 2.8 | 17 | 0.5 | nd | nd | 39 | nd |
| 2 | | | | | | | | | | |
| Lungs | 255 | 23 | 13.1 | 3.3 | 34 | 2.2 | 1.4 | nd | 25 | nd |
| Kidneys | 102 | 64 | 2.8 | 3.9 | 9 | 0.8 | nd | nd | 39 | nd |
| Liver | 87 | 520 | 2.2 | 6.4 | 15 | 1.0 | nd | nd | 49 | nd |
| Brain | 92 | nd | 0.5 | 2.6 | 21 | 0.9 | nd | nd | 26 | nd |
| Muscle | 65 | nd | nd | 2.6 | 14 | 0.6 | nd | nd | 26 | nd |
| 3 | | | | | | | | | | |
| Lungs | 144 | 266 | 7.9 | 7.9 | 16 | 1.6 | 0.2 | nd | 181 | nd |
| Kidneys | 125 | 155 | 5.1 | 5.0 | 16 | 1.8 | nd | nd | 192 | nd |
| Liver | 95 | 392 | 4.2 | 6.6 | 21 | 1.6 | nd | nd | 134 | nd |
| Brain | 65 | nd | 0.7 | 2.6 | 18 | 0.6 | nd | nd | 68 | nd |
| Muscle | 61 | 48 | nd | 4.3 | 12 | 0.5 | nd | nd | 141 | nd |
| 4 | | | | | | | | | | |
| Lungs | 153 | 87 | 10.4 | 7.2 | 30 | 1.3 | nd | nd | 64 | nd |
| Kidneys | 87 | 69 | 3.2 | 9.6 | 27 | 0.6 | nd | nd | 112 | nd |
| Liver | 120 | 419 | 3.7 | 11.5 | 35 | 2.5 | 1.3 | nd | 79 | nd |
| Brain | 84 | 72 | 0.5 | 4.8 | 20 | 0.8 | nd | nd | 48 | nd |
| Muscle | 75 | 77 | 0.6 | 7.3 | 20 | 0.7 | nd | nd | 70 | nd |
| 5 | | | | | | | | | | |
| Lungs | 172 | 76 | 11.3 | 5.2 | 16 | 1.2 | nd | nd | 72 | nd |
| Kidneys | 131 | 109 | 1.7 | 5.5 | 15 | 0.8 | nd | nd | 86 | nd |
| Liver | 164 | 367 | 2.4 | 7.7 | 16 | 0.9 | nd | nd | 73 | nd |
| Brain | 141 | 98 | 1.1 | 5.9 | 21 | 0.7 | nd | nd | 78 | nd |
| Muscle | 115 | 101 | nd | 4.8 | 16 | 0.7 | nd | nd | 85 | nd |

*NIC* nicotine, *COT* cotinine, *NNIC* nornicotine, *NCOT* norcotinine, *ABS* anabasine, *ATB* anatabine, *NNO* nicotine-*N′*-oxide, *CNO* cotinine-N-oxide, *HCOT* *trans*-3′-OH-cotinine, *MYO* myosmine, *nd* not detectable

Findings from human tissue

Data on human tissues are very limited. Neither the wide analyte pattern including major tobacco alkaloids and phase I metabolites has been covered nor has a comprehensive validation been performed in previously published studies.

Grusz-Harday [29] investigated 24 fatalities due to suicidal poisoning following oral ingestion of nicotine-containing insecticidal preparations. In all cases, NIC could be detected in the liver at concentrations ranging from 4 to 2270 mg/kg; in 20 cases, NIC could also be found in the brain, whereas kidney and lung tissues were NIC positive in only 17 and 2 specimens, respectively. In gastric contents, fairly large quantities of up to 155 g NIC were present, suggesting that death had occurred very rapidly following uptake of the nicotine-containing formulation. Therefore, incomplete distribution of the toxic agent at the time of death is a likely explanation for the findings of Grusz-Harday [29] and also for the different distribution patterns experienced from the present results.

Kemp et al. [30] found NIC and COT concentrations of the same order of magnitude in the brain from a death case attributed to suffocation having 18 NIC patches taped to the chest and the abdomen, in addition. In the present study, the brain concentrations of NIC in case nos. 1 and 4 did not differ significantly from those of COT; it amounts to 144 % of the respective COT concentration in case no. 5, whereas COT was not detectable in case nos. 2 and 3. This may indicate toward NIC crossing the blood-brain barrier more efficiently than COT. This assumption has been verified in an animal model by Riah et al. [31].

Kemp et al. [30] estimated the liver NIC/COT concentration ratio at 0.52. In our study, respective concentration ratios

ranged from 0.17 to 0.46. It may be speculated whether the higher ratio reported by Kempf et al. [30] is due to a rapid death or to a penetration of NIC through the skin during the postmortem phase from the patches or from a transient skin depot due to stratum corneum retention of the drug [32].

Urakawa et al. [33] observed concentrations of NIC and COT in liver specimens from ten habitual smokers ranging from 14 to 325 ng/g and from 260 to 1586 ng/g, respectively. These ranges are considerably broader than those observed in the present study (NIC 87–164 ng/g, COT 367–520 ng/g) whereas lung concentrations of NIC and COT as well as kidney concentrations of COT were comparable. However, kidney concentrations of NIC showed a broader span from 87 to 1075 ng/g in the present investigation as a result of the high NIC concentration seen in case no. 1. Likewise, the analytes' concentrations except HCOT were highest in the kidney tissue of case no. 1 compared to case nos. 2–5. Concentration ranges of NIC and COT in the brain and muscle specimens also differed in both studies; currently, the small sample size does not allow further interpretation. To date, no reference data determined from human tissue are available for NNIC, NCOT, ABS, ATB, NNO, CNO, HCOT, and MYO.

The highest concentrations of NNIC and NCOT were present in the lungs, liver, and kidneys compared to the brain and muscle tissues. Especially, NNIC could not be detected in the muscle tissue in four out of the five cases; its concentration was always lower in brain specimens (≤1.1 ng/g) compared to NCOT. NCOT as well as ATB and ABS could be identified in all tissue specimens obtained from case nos. 1–5 at about 10- to 30-fold higher concentrations of ABS compared to ATB. Mean elimination half-lives of ABS and ATB are 15.9 h and 9.6 h, respectively, thus explaining the present ABS findings to some extent [2]. However, ATB concentrations being 4- to 9-fold higher in all tobacco products investigated by Jacob et al. [2] is not in line with this observation.

*N*-Oxide metabolites are commonly prone to degradation, especially by reduction to the parent compound. In vivo experiments led to detection of NNO in cat liver after intravenous administration of radiolabeled NIC [34]. Booth and Boyland [35] observed formation of NNO in liver, kidney, and brain samples incubated with NIC. However, tissue binding of NIC appears to depend on the particular species. Comparing rat, cat, and pigeon tissues, Leeds and Turner [36] observed a low binding capability of NIC to cat liver, but a higher one to rat kidney. With regard to the proven metabolization of NIC to NNO in mammalian tissues, negative findings of NNO and CNO in the present investigation are assumed to be due to degradation during the postmortem interval or during sample storage; freeze-thaw instability may have contributed to further degradation of *N*-oxides.

MYO was not detectable in any of the investigated tissue samples, which suggests still lowering its LLOD. Schutte-Borkovec [37] found quite low mean MYO concentrations of 0.155 ng/mL and 0.296 ng/mL in plasma of nonsmokers and smokers, respectively. Due to the freeze-thaw instability of some analytes, inter-day precision experiments can only be promising using freshly prepared QC controls. In addition, including phase II metabolites in further investigations will provide additional information on their distribution in human specimens.

## Conclusion

For all analytes except MYO, an assay combining efficient extraction with sensitive and selective quantifications has been established in a small amount of tissue homogenate with a high coverage of genuine tobacco alkaloids and major phase I metabolites as well as an unambiguous LC separation of isobaric analyte pairs. Freeze-thaw instability of some analytes has still to be followed up as well as advanced stability experiments in human tissues.

Comparing blood, urine, and hair data to advanced tissue data in a future study may be helpful to classify the latter ones for forensic case work. Such information will also enable to answer requests of insurance companies as indicated in the introduction when only tissue specimens are available.

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